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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/52, 15/76, 1/21, C07D 493/08 // (C07D 493/08, 321:00, 311:00)

A1

(11) International Publication Number: **WO 99/36546**

(43) International Publication Date: 22 July 1999 (22.07.99)

(21) International Application Number: PCT/GB99/00117

(22) International Filing Date: 14 January 1999 (14.01.99)

(30) Priority Data:

| | | |
|---|---|---|
| 9800675.2 | 14 January 1998 (14.01.98) | GB |
| 9800879.0 | 15 January 1998 (15.01.98) | GB |


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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: POLYKETIDES AND THEIR SYNTHESIS

(57) Abstract

The present invention provides an extended polyketide synthase gene assembly comprising a loading module and a plurality of extension modules wherein a second nucleic acid portion, encoding at least one extension module, has been inserted into a first nucleic acid portion encoding the loading module and at least one, and preferably a plurality, of extension modules. Such extended genes are useful in the production of novel macrolides of larger size than the natural product, for example 16-membered ring macrolides instead of 14-membered ring macrolides.

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# Polyketides and their Synthesis

The present invention relates to processes for preparing novel polyketides, particularly 16-membered ring macrolides, by recombinant synthesis and to the novel polyketides so produced. Polyketide biosynthetic genes or portions of them, which may be derived from different polyketide biosynthetic gene clusters are manipulated to allow the production of specific novel polyketides, such as 16-membered ring macrolides, of predicted structure. The invention is particularly concerned with the incorporation of additional genes in order to prepare macrolides of larger size than the natural product, for example 16-membered ring instead of 14-membered ring macrolides.

Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin and FK506. In particular, polyketides are abundantly produced by Streptomyces and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the β-keto group observed after each condensation. Examples of processing steps include reduction to β-hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of chain extension.

The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS) have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, avermectin and rapamycin (Figure 1), consists of a different set or "module" of enzymes for each cycle of polyketide chain extension (Figure 2) (Cortes, J. *et al.* Nature (1990) 348:176-178; Donadio, S. *et al.* Science (1991) 252:675-679; MacNeil, D.J. *et al.* Gene (1992)

115:119-125; Schweke, T. *et al.* Proc. Natl. Acad. Sci USA (1995) 92:7839-7843).

The term "natural module" as used herein refers to the set of contiguous domains, from a β-ketoacyl-ACP synthase ("KS") gene to the next acyl carrier protein ("ACP") gene, which accomplishes one cycle of polyketide chain extension. The term "combinatorial module" is used to refer to any group of contiguous domains (and domain parts), extending from a first point in a first natural module, to a second equivalent point in a second natural module. The first and second points will generally be in core domains which are present in all modules, ie both at equivalent points of respective KS, AT (acyl transferase) or ACP domains.

The length of polyketide formed has been altered, in the case of erythromycin biosynthesis, by specific relocation using genetic engineering of the enzymatic domain of the erythromycin-producing PKS that contains the chain releasing thioesterase/cyclase activity (Cortes, J *et al.* Science (1995) 268:1487-1489; Kao, C. M. *et al.* J. Am. Chem. Soc. (1995) 117:9105-9106).

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin producing PKS, (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3-$\alpha$-mycarosyl-5-oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy-6, 6$\beta$-epoxy-5-oxoerythronolide B (Donadio, S. et al. Science, (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into Saccharopolyspora erythraea, led to the production of 6,7-anhydroerythromycin C (Donadio S. *et al.* Proc. Natl. Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However, many such attempts are reported to have been unproductive (Hutchinson C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p.231).

The complete DNA sequence of the genes from Streptomyces hygroscopicus that encode the modular Type 1 PKS governing the biosynthesis of the macrocyclic immunosuppressant polyketide rapamycin has been disclosed (Schwecke, T. *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843). The DNA sequence is deposited in the EMBL/Genbank Database under the accession number X86780.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used as appropriate in successive cycles (Bibb, M. J. *et al.* EMBO J. (1989) 8:2727-2736; Sherman, D.H. *et al.* EMBO J. (1989) 8:2717-2725; Fernandez-Moreno, M.A. *et al.* J. Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II PKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained by the introduction of cloned Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from Streptomyces coelicolor, into an anthraquinone polyketide-producing strain of Streptomyces galileus (Bartel, P. L. *et al.* J. Bacteriol. (1990) 172:4816-4826).

International Patent Application Number WO 95/08548 describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS clusters, to obtain hybrid polyketides. The same International Patent Application WO 95/08548 describes the construction of a strain of Streptomyces coelicolor which substantially lacks the native gene cluster for actinorhodin, and the use in that strain of a plasmid vector pRM5 derived from the low-copy number plasmid vector SCP2* isolated from Streptomyces coelicolor (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and in which heterologous PKS-containing DNA may be expressed under the control of the divergent act I/act III promoter region of the actinorhodin gene cluster (Fernandez-Moreno, M. A. *et al.* J.Biol. Chem. (1992) 267:19278-19290). The

plasmid pRM5 also contains DNA from the actinorhodin biosynthetic gene cluster encoding the gene for a specific activator protein, Act II-orf4. The Act II-orf4 protein is required for transcription of the genes placed under the control of the act I/act III bidirectional promoter and activates expression during the transition from growth to stationary phase in the vegetative mycelium (Hallam, S. E. *et al.* Gene (1988) 74:305-320).

Type II PKS clusters in Streptomyces are known to be activated by pathway-specific activator genes (Narva, K. E. and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333; Stutzman-Engwall, K. J. *et al* J. Bacteriol (1992) 174:144-154; Fernandez-Moreno, M. *et al.* Cell (1991) 66:769-780; Takano, E. *et al.* Mol. Microbiol. (1992) 7:837-845; Takano, E. *et al.* Mol. Microbiol (1992) 6:2797-2804) whose gene product is required for transcription from specific promoters. The gene product of the activator genes is speculated to act by binding to specific DNA sequences in promoters of the PKS gene cluster in which the activator gene is located (Stutzman-Engwall, K. J. *et al.* J. Bacteriol (1992) 174:144-154; Takano, E. *et al.* Mol. Microbiol. (1992) 7:837-845). The DnrI gene product complements a mutation in the actII-orf4 gene of S. coelicolor, implying that DnrI and ActII-orf4 proteins act on similar targets. A gene (srmR) has been described (EP 0 524 832 A2) that is located near the Type I PKS gene cluster for the macrolide polyketide spiramycin. This gene specifically activates the production of the macrolide polyketide spiramycin, but no other examples have been found of such a gene. Also, no homologues of the ActII-orf4/DnrI/RedD family of activators have been described that act on Type I PKS genes.

Although large numbers of therapeutically important polyketides have been identified, there remains a need to obtain novel polyketides that have enhanced properties or possess completely novel bioactivity. The complex polyketides produced by modular Type I PKSs are particularly valuable, in that they include compounds with known utility as antihelminthics, insecticides, immunosuppressants, antifungal or antibacterial agents. Because of their structural complexity, such novel polyketides are not readily obtainable by total chemical synthesis, or by chemical modifications of known polyketides.

There is also a need to develop reliable and specific ways of deploying individual modules in practice so that all, or a large fraction, of hybrid PKS genes that are constructed, are viable and produce the desired polyketide product.

Pending International Patent Application no. PCT/GB97/01819 discloses that a PKS gene assembly (particularly of type I) encodes a loading module which is followed by at least one extension module. Thus Fig 2a shows the organisation of the DEBS genes. The first open reading frame encodes the first multi-enzyme or cassette (DEBS 1) which consists of three modules; the loading module (ery-load) and two extension modules (Modules 1 and 2). The loading module comprises an acyl transferase and an acyl carrier protein. This may be contrasted with Fig. 1 of WO93/13663 (referred to above). This shows ORF1 to consist of only two modules, the first of which is in fact both the loading module and the first extension module.

PCT/GB97/01819 describes in general terms the production of a hybrid PKS gene assembly comprising a loading module and at least one extension module. However it contains no specific disclosure of the preparation of a PKS gene assembly to which extra modules, over the number which would be present in the natural PKS assembly, have been added.

Marsden, A. F. A. *et al.* Science (1998) Vol 279 5348:199-202 describes construction of a hybrid PKS gene assembly by grafting the wide-specificity loading module for the avermectin-producing polyketide synthase onto the first multienzyme component (DEBS 1) for the erythromycin PKS in place of the normal loading module. Certain novel polyketides can be prepared using the hybrid PKS gene assembly.

Katz, L. Chem Rev. (1997) 7:2557-2575 states that chain length of a polyketide can be changed by removing or adding modules, but that only removal of modules, resulting in the shortening of the PKS chain, has been disclosed. It has now been found that modules can be added to PKS gene assemblies and that th se lengthened assemblies can be successfully used to prepare polyketides having a longer chain length than the natural product.

In one aspect, the present invention concerns the production of an extended PKS gene assembly comprising a loading module and a plurality of extension modules by inserting a second nucleic acid portion, encoding at least one extension module, into a first nucleic acid portion encoding the loading module and at least one, and preferably a plurality, of extension modules. Generally the nucleic acids are DNA.

Preferably the extended PKS gene encodes a loading module and from 2 to 7, extension modules. Suitably the first portion encodes a loading module and 1 to 6 extension modules and a chain terminating enzyme (generally a thioesterase) and the second portion encodes an extension module.

Preferably, the PKS gene encodes a Type I PKS.

It is particularly useful to insert an extension module into a PKS gene assembly which would otherwise produce a 14-membered ring macrolide in order to produce an extended PKS assembly which can be used to prepare a 16-membered ring macrolide. Particularly suitable PKSs for this purpose are the components of PKSs for the biosynthesis of erythromycin, rapamycin, avermectin, tetronasin, oleandomycin, spiromycin, tylomycin, FK506, midecamycin and rifamycin, for all of which the gene and modular organisation is known at least in part.

The extension module or modules inserted into the first nucleic acid portion may be homologous or heterologous. They may be natural sequences derived from the same or from a different source eg the first portion may encode modules of erythromycin PKS and the second portion may encode an extension module of a different PKS such as avermectin. Alternatively the second portion may encode a modified extension module which may be an altered version of a natural module from the same or a different source than the first portion.

The second portion may be entirely synthetic or may be an engineered, eg by splicing, module comprising DNA derived from one or more different sources.

The first portion may encode a hybrid PKS produced, for example, as described in PCT/GB97/01819. It may even have been produced by prior insertions of extension modules. Thus this aspect of the invention encompasses stepwise addition of a plurality of nucleic acid sequences, wherein each sequence encodes at least one extension module, into an initial nucleic acid portion encoding a loading module and at least one extension module. Suitably, the loading module encoded in the first portion shows a relaxed specificity, for example the loading module of the avermectin *(avr)*-producing PKS of streptomyces avermitilis; or those loading modules possessing an unusual specificity, for example the loading modules of the rapamycin, FK506- and ascomycin-producing PKSs, all of which naturally accept a shikimate-derived starter unit.

Most preferably the second portion of nucleic acid is inserted between the nucleic acid which encodes the first and second extension modules in the first portion. Alternatively the second portion of nucleic acid may be inserted between the nucleic acid which encodes the final extension module and that encoding the chain terminating enzyme in the first portion.

The end of the gene assembly may also be altered. Thus the normal chain terminating enzyme of a PKS (usually thioesterase) may be replaced by an enzyme leading to a different type of product. For example, use may be made of the enzyme from the rapamycin system that connects the polyketide chain to an aminoacid or, possibly, an aminoacid chain. The latter can be used to synthesise polypeptide/polyketide combinations, eg for producing $\beta$-lactam derivatives.

This aspect of the invention is largely concerned with treating PKS gene modules as building blocks and inserting additional blocks into existing assemblies. It might be assumed that the correct places for making and breaking the intermodular connections would be in the linking regions between modules, where previously reported experiments using limited proteolysis have shown those linkers to be on the surface of the protein (Aparicio, J. F. *et al.* (1994) J. Biol. Chem. 269:8524-8528; Staunton J. *et al.* (1996) Nature Structure Biol 3:188-192). However it has been found that it may sometimes be preferable

to make cuts and joins actually within domains (i.e. the enzyme-coding portions), close to the edges thereof. The DNA is highly conserved here between all modular PKSs and this may aid in the construction of new extended PKS assemblies that can be transcribed.

The invention further provides such extended PKS assemblies, vectors containing such gene assemblies, and transformant organisms that can express them. Transformant organisms may harbour recombinant plasmids, or the plasmids may integrate. A plasmid with an *int* sequence will integrate into a specific attachment site *(att)* of a host's chromosome. Transformant organisms may be capable of modifying the initial products, eg by carrying out all or some of the biosynthetic modifications normal in the production of erythromycins (as shown in Fig 2b) and other polyketides. Use may be made of mutant organisms such that some of the normal pathways are blocked, eg to produce products without one or more 'natural' hydroxy-groups or sugar groups. The invention further provides novel polyketides as producible, directly or indirectly, by transformant organisms. (This includes polyketides which have undergone enzymic modification).

In a further aspect the invention provides novel polyketides obtainable by means of the previous aspects. These include 16-membered ring macrolides which are extended homologues of polyketides, such as erythromycin (and analogues and other derivatives thereof), which naturally exist as 14-membered rings. Novel derivatives of known 16-membered macrolides, such as tylosin and rokitamycin (or the leucomycin A5 precursor of rokitamycin) analogues, may also be produced by adding an extension module to PKS assemblies which would naturally produce 14-membered macrolides. These may differ from the corresponding 'natural' compound:

a) in the oxidation state of one or more of the ketide units (ie selection of alternatives from the group: -CO-, -CH(OH)-, =CH-, and $-CH_2-$) where the stereochemistry of any -CH(OH)- is also independently selectable; or

b) in the absence of a 'natural' methyl side-chain; or

c) in the stereochemistry of 'natural' methyl; and/or ring substituents other than methyl.

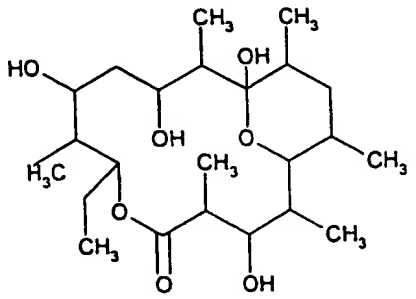
It is also possible to prepare analogues of 16-membered macrolides such as tylosin or rokitamycin (particularly the leucomycin A5 precursor of rokitamycin) having the differences from the natural product identified in two or more of items (a) to (c) above.

Longer homologues of polyketides of types other than erythromycin, optionally having additional modifications such as are proposed for tylosin and rokitamycin (or leucomycin A5) above, may also be prepared. Examples of polyketides of which such longer homologues may be prepared include rapamycin, avermectin, tetronasin, oleandomycin, monensin, amphotericin and rifamycin.

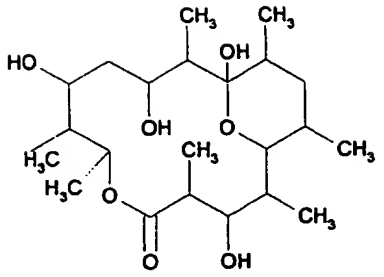
Ketide/non-ketide fusions may be prepared, as may polyketides (or fusions) cyclised by formation of lactones, hemiketals, ketals, lactams or lactols.

Derivatives of any of the aforementioned polyketides which have undergone further processing by non-PKS enzymes, eg one or more of hydroxylation, epoxidation, glycosylation and methylation may also be prepared.

Examples of preferred compounds according to the present invention are those of formulae (I) and (II)




(I)



(II)

The present invention provides a method of obtaining novel complex polyketides; and novel methods of producing both known and novel polyketides. In preferred embodiments, the invention provides novel 16-membered macrolides, for example the compounds of formulae (I) and (II), and a new way of producing both those novel compounds and known compounds such as rokitamycin (or leucomycin A5) and tylosin. Other 16-membered macrolides which may be prepared by the method of the present invention include josamycin, spiramycin, miokamycin, leucomycin and midecamycin. The preferred method for making such 16-membered macrolides is to insert DNA encoding an additional extension module into a PKS gene assembly which would normally be expected, when expressed, to produce a 14-membered macrolide.

It is particularly useful to be able to prepare a range of 16-membered macrolides as such compounds are generally considered to have a better side-effect profile, when used as medicaments, than 14-membered macrolides.

It might have been expected that the chain terminating enzyme of an extended PKS assembly produced according to the present invention would tend to cyclise the polyketide chain so as to produce a macrolide having the natural number of ring members. For example, where an additional extension module is inserted into an erythromycin assembly, it might be predicted that the erythromycin thioesterase would continue to attack the group added by module 6 so as to produce a 14-membered macrolide. However, it has unexpectedly and surprisingly been found that the extended PKS gene assemblies do produce macrolides with extended rings, i.e. in the case of erythomycin, a measurable quantity of 16-membered ring macrolide is produced. This means that ext nd d

PKS assemblies can be used to prepare larger than natural macrolides. For example, new rokitamycin (or leucomycin A5) analogues can conveniently be made from modified erythromycin gene assemblies.

Suitable plasmid vectors and genetically engineered cells suitable for expression of extended PKS genes are those described in PCT/GB97/01819 as being suitable for expression of hybrid PKS I genes. Examples of effective hosts are Saccharopolyspora erythraea, Streptomyces coelicolor, Streptomyces avermitilis, Streptomyces griseofuscus, Streptomyces cinnamonensis, Micromonospora griseorubida, Streptomyces hygroscopicus, Streptomyces fradiae, Streptomyces longisporoflavus, Streptomyces lasaliensis, Streptomyces tsukubaensis, Streptomyces griseus, Streptomyces venezuelae, Streptomyces antibioticus, Streptomyces lividans, Streptomyces rimosus and Streptomyces albus. These include hosts in which SCP2*-derived plasmid vectors are known to replicate autonomously, such as for example S. coelicolor, S. avermitilis and S. griseofuscus; and other hosts such as Saccharopolyspora erythraea in which SCP2*-derived plasmids become integrated into the chromosome through homologous recombination between sequences on the plasmid insert and on the chromosome; and all hosts which are integratively transformed by suicide plasmid vectors.

In a further aspect of the present invention, a plasmid containing "donor" PKS DNA is introduced into a host cell under conditions where the plasmid becomes integrated into an acceptor PKS gene assembly on the bacterial chromosome to create an extended PKS. A preferred embodiment is when the donor PKS DNA includes a segment encoding an extension module.

Some embodiments of the invention will now be described with reference to the accompanying drawings in which:

Fig 1 gives the chemical formulae of known polyketides.

Fig 2a is a diagram showing the functioning of 6-deoxyerythrondide synthase B (DEBS), a PKS producing 6-deoxyerythronolide B (6-DEB), a precursor of erythromycin A;

Fig 2b shows post-PKS biosynthesis of erythromycins including the conversion of 6-DEB to erythromycin A

The present invention will now be illustrated, but is not intended to be limited, by means of some examples.

Example 1
Construction of the Recombinant Vector pCJR54

Plasmid pCJR24 was prepared as described in PCT/GB97/01819. Restriction digestion, kinase end polishing and re-ligation techniques were used to remove the Sse8387I polylinker site from pCJR24 to give plasmid pCJR24-S.

An Sse8387I restriction site was created 6120 base pairs into the DEBSI-TE encoding gene using a polymerase chain reaction (PCR) strategy. The base changes which created this site did not introduce any amino acid mutations.

The DEBSI-TE encoding gene containing the Sse8387I site was introduced into pCJR24-S to give plasmid pIB103.

Rapamycin module 2 was isolated as a Sse8387I fragment by restriction digestion. Changes to the DNA sequence to introduce sites was achieved using PCR and did not introduce any amino acid mutations. Rapamycin module 2 was taken from the amino acid sequence (ACR) at 9900 bases from the start of the *rap*A gene to the ACR at 14633 bases from the start of *rap*A.

The resulting 4763 bp fragment of DNA containing the modified rapamycin module 2 was inserted as an Sse8387I fragment into Sse8387I-cut pIB103 to yield pCJR54.

Example 2
Construction of S.erythraea NRRL2338/no.5/pCJR54

S.erythraea was tranformed with pCJR54 (transformation into S.erythraea is described in PCT/GB97/01819) by homologous recombination. Selection of

correct integrants was verified by selection of thiostrepton resistant clones. A single clone, no.6, was selected. S. erythraea NRRL2338/No. 5 was selected for its inability to glycosylate the erythromycin agtycone.

Example 3

Production of polyketides using S.erythraea NRRL 2338/no.5/pCJR54/6

A frozen suspension of strain pCJR54/no.5/6 was used to inoculate 50ml of SV2 medium in a 250ml conical flask. SV2 contained, per litre deionised water, glucose 15g, glycerol 15g, soy peptone 15g, NaCl 3g, $CaCO_3$ 1g. The pH was adjusted to 7.0 pre-autoclaving. Thiostrepton was added pre-inoculation to give a concentration of 5μg $mL^{-1}$. This seed culture was shaken at 250rpm, 28°C, for 3 days and then used to inoculate the production medium SM3. The production medium contained, per litre deionised water, glucose 5g, maltodextrin (glucidex) 50g, soya flour (arkasoy) 25g, beet molasses 3g, $K_2HPO_4$ 0.25g, $CaCO_3$ 2.5g. The pH was adjusted to 7.0 pre-autoclaving. Thiostrepton was added to give a concentration of 5μg $mL^{-1}$ before inoculation. The seed culture was used to inoculate (2% volume/volume) seven 2-litre florence flasks containing 300mL of SM3, which were shaken at 250rpm and 28°C for 7 days before harvest. The fermentation broth (2L) was centrifuged at 4500rpm. The cells and supernatant were separated by decantation and the pH of the supernatant was adjusted to pH9.5. The resulting solution was extracted with ethylacetate (2L). The ethylacetate layer was removed and evaporated under vacuum to yield 1.297g of oil.

Ethylacetate (0.4mL), methanol (0.8mL), 10mM ammonium acetate (1.2mL) and 80:20, $CH_3CN$: 20mM ammonium acetate (0.8mL) were added to the oil and the resulting solution was subjected to preparative HPLC using the conditions set out below:

| | |
|---|---|
| Column | 7 micron Kromasil C8 (6cm x 2.5cm i.d.) connected in series with a 5 micron Hypersil C18 BDS (15cm x 2.5cm i.d.) |
| Mobile Phases | A. 10mM Ammonium Acetate |

B. 80:20 $CH_3CN$:20mM Ammonium Acetate

| Gradient Program | Time (mins) | %B | Flow Rate (mL/min) |
|---|---|---|---|
| | 0 | 0 | 20 |
| | 1 | 0 | 20 |
| | 60 | 75 | 20 |

The eluent was monitored by UV spectroscopy at 210nm and two successive separations, each on 1.8mL of solution (prepared as described above) were carried out. Fractions were collected at 0.5 minute intervals and (10mL volumes) and subjected to Mass spectrometry (-ve APCI).

Isolation of compound of formula (I)

Fractions containing a compound of mass 430 eluting between retention times of 48 to 50.5 minutes were bulked from the above preparative separations and the $CH_3CN$ removed under a stream of nitrogen. The aqueous solution was passed through an iso-elut env+ cartridge (500mg) (International Sorbent Technology). The cartridge was washed with distilled water and eluted with MeOH (2.5mL). The MeOH was removed by centrifugal evaporation and the solid (1.5mg) isolated. This was identified as the compound of formula (I).

A 500 MHz proton NMR spectrum of a solution in hexadeutero dimethyl sulphoxide at about 323°K includes peaks [δ values with multipicities, coupling constants (Hz) and integration values in parentheses] centered at about:

0.75(d,6.5,3H), 0.93(d,6.5,3H), 2.21(m,1H), 2.74(m,1H), 3.44(dd,10.5,2,1H), 3.55(t,9.5,1H), 3.85(dd,10.5,3.5,1H), 3.93(dd,9,2,1H), 4.74(m,1H).

Isolation of compound of formula (II)

Fractions containing a compound of mass 416 eluting between retention times of 43.5 to 45 minutes were bulked from the initial preparative separations and the $CH_3CN$ removed under a stream of nitrogen. The aqueous solution was passed through an iso-elut env+ cartridge (500mg). The cartridge was washed with

distilled water and eluted with MeOH (2.5mL). The MeOH was removed by centrifugal evaporation and a solid (117.5mg) recovered. The solid was dissolved in MeOH (0.9mL) 10mM ammonium acetate (0.6mL) and this solution was subjected to preparative HPLC using the conditions listed below:-

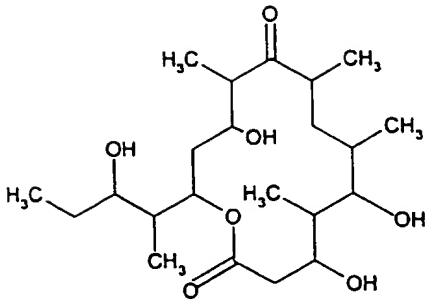
| | |
|---|---|
| Column | 5 micron Hypersil C18 BDS (15cm x 2.5 cm i.d.) |
| Mobile Phases | As above |
| Gradient Program | As above |

The eluent was monitored by UV spectroscopy at 210nm and fractions were collected at 0.5 minute intervals (10mL volumes) and subjected to Mass spectroscopy (-ve APCI). Fractions containing the compound of mass 416 eluting between retention times of 38.5 to 40 minutes were bulked. The $CH_3CN$ was removed under a stream of nitrogen and the aqueous solution was passed through an iso-elut env+ cartridge (500mg). The cartridge was washed with distilled water and eluted with MeOH (2.5mL). The MeOH was removed by centrifugal evaporation and a solid (1.5mg) isolated. This was identified as the compound of formula (II).

A 500 MHz proton NMR spectrum of a solution in hexadeutero dimethyl sulphoxide includes peakes [δ values with multipicities, coupling constants (Hz) and integration values in parentheses] centred at about:

0.71(d,6.5,3H), 0.80(d,7,3H), 0.82(d,6.5,3H), 0.90(d,6.5,6H), 0.93(d,7,3H), 1.07(d,6.5,3H), 1.71(m,1H), 2.22(dq,3.5,7,1H), 3.54(m,1H), 3.88(m,1H), 3.99(m,1H), 4.75(m,1H).

Isolation of Compound of Formula (III)



(III)

Fractions containing a compound of mass 416 eluting between 32.5 to 33.5 were bulked from the initial preparative separations and the $CH_3CN$ removed under a stream of nitrogen. The aqueous solution was passed through an iso-elut env+ cartridge (500mg). The cartridge was washed with distilled water and eluted with MeOH (2.5mL). The MeOH was removed by centrifugal evaporation and a solid (4.1mg) isolated. This solid was dissolved in DMSO (0.2mL), MeOH (0.4mL) and 10mM ammonium acetate (1.0mL) and subjected to preparative HPLC using the conditions as listed for the isolation of the compound of formula (II). Fractions containing the compound of mass 416 eluting between 29.5 to 30.5 were bulked and the $CH_3CN$ removed under a stream of nitrogen. The aqueous solution was passed through an iso-elut env+ cartridge (500mg). The cartridge was washed with distilled water and eluted with MeOH (2.5mL). The MeOH was removed by centrifugal evaporation and a solid (0.2mg) isolated. This was identified as the compound of formula (III).

A 500MHz proton NMR spectrum of a solution in hexadeutero dimethyl sulphoxide includes peaks [δ values with multipicities, coupling constants (Hz) and integration values in parentheses] centred at about:

0.82(ddd,5.5,11,14.5,1H), 0.91(d,7,3H), 0.92(d,7,3H), 0.93(d,7,3H), 0.97(d,6.5,3H), 0.99(d,7,3H), 1.04(d,6,3H), 1.10(d,6.5,3H), 1.49(m,1H), 1.77(ddd,3.5,6,14.5,1H), 2.58(dq,9.5,6.5,1H), 3.94(m,1H), 5.23(ddd,2,4.5,10,1H).

Isolation of Erythronolide B

Fractions of mass 402 eluting between 37.5 to 38.5 were bulked from the initial preparative separations and the $CH_3CN$ removed under a stream of nitrogen. The aqueous solution was passed through an iso-elut env+ catridge (500mg). The cartridge was washed with distilled water and eluted with MeOH(2.5ml). The MeOH was removed by centrifugal evaporation and a solid (17.5mg) identified as erythronolide B.

Example 4
Construction of Recombinant Vector pIB126

Plasmid pIB126 was constructed as follows:

A *Nhe*I restriction site was created at 6063 bp into the DEBS1-TE gene using a polymerase chain reaction (PCR) strategy. The introduction of the site resulted in amino acid change from QAA to QLA.

The DEBS1-TE gene containing the *Nhe*I site was cloned into pCJR24 to give pIB117.

The rapamycin polyketide synthase module 5 was inserted into the *Nhe*I site in the linker region between ACP1 and KS2 from the DEBS1-TE gene. Changes to introduce a *Nhe*I site at either end of the rapamycin module 5 were introduced by PCR. This resulted in amino acid changes from AN to LA at the beginning and RT to LA at the end of the module, respectively. The rapamycin module 5 was taken from position 67 bp (AN) to position 4873 bp (RT) from the start of *rap*B. The resulting 4854 bp fragment, containing module 5 with *Nhe*I restriction sites at either end was inserted into pIB117 at the *Nhe*I site to yield plasmid pIB126.

Example 5
Construction of S. *erythraea* NRLL2338/pIB126

S. *erythraea* was transformed with pIB126. Integration of the plasmid into the chromosome was verified by selection of thiostrepton resistant clones. A single clone was selected.

Example 6
Production of polyketides using S. erythraea NRLL2338/pIB126

A frozen suspension of S. erythraea NRLL2338/pIB126 was inoculated into TSB medium containing thiostrepton (5 ug/ml). This seed culture was shaken at 250 rpm, 28°C, for 3 days and then used to inoculate the production medium SM3. The remaining fermentation steps were as described hereinbefore for Example 3.

A sample of extract from the fermentation of S. *erythraea* NRRL2338/pIB126 was analysed by electrospray (ES) mass spectroscopy (MS), with verification by retention time and ion masses. Authentic samples of compound I and compound II obtained from S. *erythraea* NRRL2338/no.5/pCJR54/no.6 were run as standards.

HPLC conditions:
Buffer A: 10 mM Ammonium acetate + 0.1% formic acid
Buffer B: 90% Acetonitrile/10% water; 10 mM ammonium acetate + 0.07% formic acid

Column: Hypersil $C_{18}$ BDS, 3 micron, 150 x 4.6 mm.
Flow Rate: 1 mL/min
Gradient:

| | | | |
|---|---|---|---|
| 0.00 min | | 98%A | 2%B |
| 2.00 min | | 98%A | 2%B |
| 20.00 min | 2%A | 98%B | |
| 27.50 min | 2%A | 98%B | |
| 28.00 min | 98%A | 2%B | |
| 32.00 min | 98%A | 2%B | |

Compound I : Retention time = 16.35 min
Compound II : Retention time = 15.35 min

Electrospray (ES) mass spectroscopy (MS) was performed in both +ve and -ve modes. Ions were observed in +ve mode as $MH^+$ and $MH^+-H_20$ adducts. Ions were observed in -ve mode as $[M-H]^-$ or $[M(formate)]^-$.

Although the present invention is illustrated by the examples listed above, they should not be regarded as limiting the scope of the invention. The above descriptions illustrate, for the first time the construction of an extended Type I PKS gene assembly, by insertion of an additional extension module and its use to obtain novel polyketide products of utility as synthetic intermediates or as bioactive materials such as antibiotics. Insertion of a rap extension module into an ery PKS has been described herein to obtain novel 16-membered erythromycin homologues. It will readily occur to the person skilled in the art that extension modules from other PKS gene sets could be inserted into an ery PKS, or, indeed into a quite different PKS gene assembly.

## Claims

1. An extended polyketide synthase (PKS) gene assembly comprising nucleic acid encoding a loading module and a plurality of extension modules wherein a second nucleic acid portion encoding at least one extension module has been inserted into a first nucleic acid portion encoding the loading module and at least one extension module.

2. An extended PKS gene assembly according to claim 1 wherein the first nucleic acid portion encodes a PKS gene assembly which can be used to produce a 14-membered macrolide when expressed and the extended PKS gene assembly can be used to produce a 16-membered macrolide when expressed.

3. An extended PKS as encoded by a gene assembly according to claim 1 or claim 2.

4. A vector including a gene or nucleic acid according to claim 1 or claim 2.

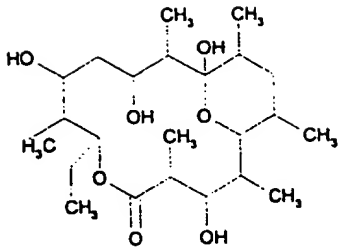
5. A transformed organism containing a gene or nucleic acid according to claim 1 or claim 2 and able to express a polyketide synthase encoded thereby.

6. A method of making a polyketide by culturing the organism of claim 5.

7. A polyketide as prepared by the method of claim 6.

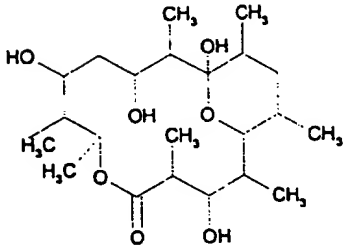
8. A polyketide according to claim 7 which is a 16-membered macrolide.

9. A polyketide of formula I



(I)

or formula II



(II)

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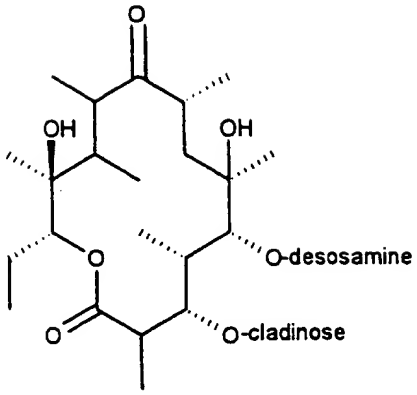
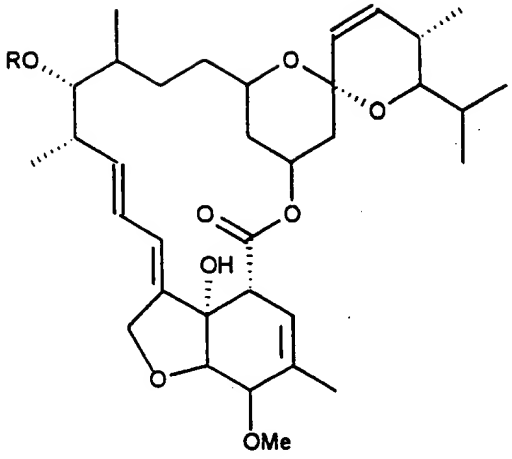
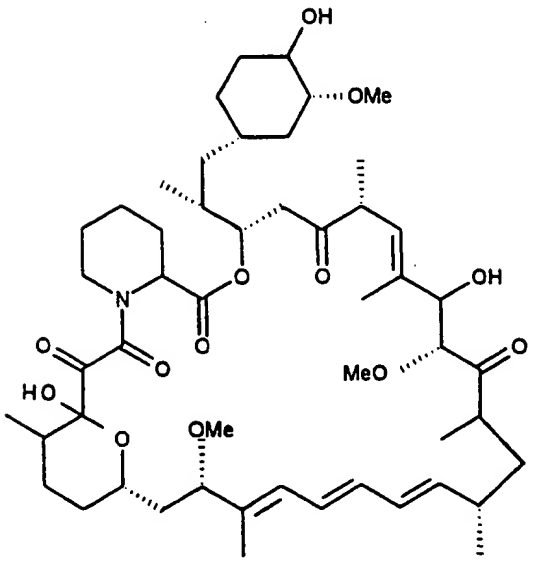


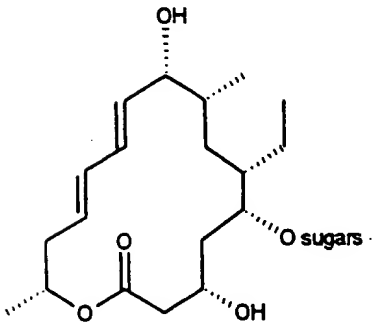

Erythromycin A



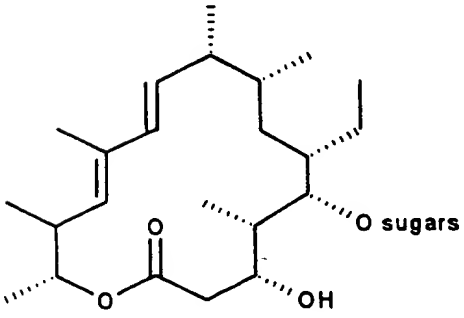

Avermectin A1b




Rapamycin




Rokitamycin




Tylosin

FIG. 1

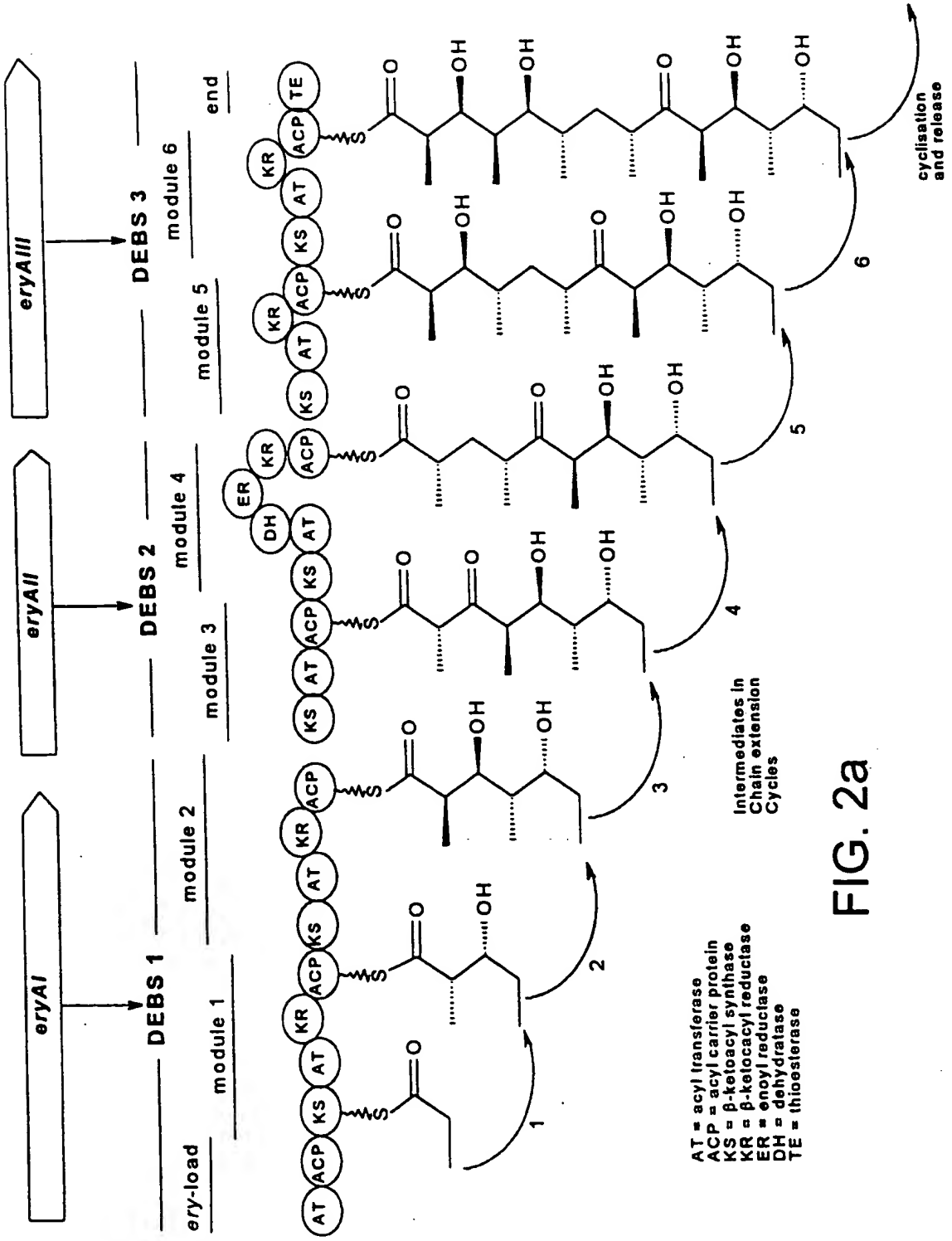

eryAI
eryAII
eryAIII
DEBS 1
DEBS 2
DEBS 3
ery-load
module 1
module 2
module 3
module 4
module 5
module 6
end
AT = acyl transferase
ACP = acyl carrier protein
KS = β-ketoacyl synthase
KR = β-ketoacyl reductase
ER = enoyl reductase
DH = dehydratase
TE = thioesterase
Intermediates in Chain extension Cycles
cyclisation and release

FIG. 2a

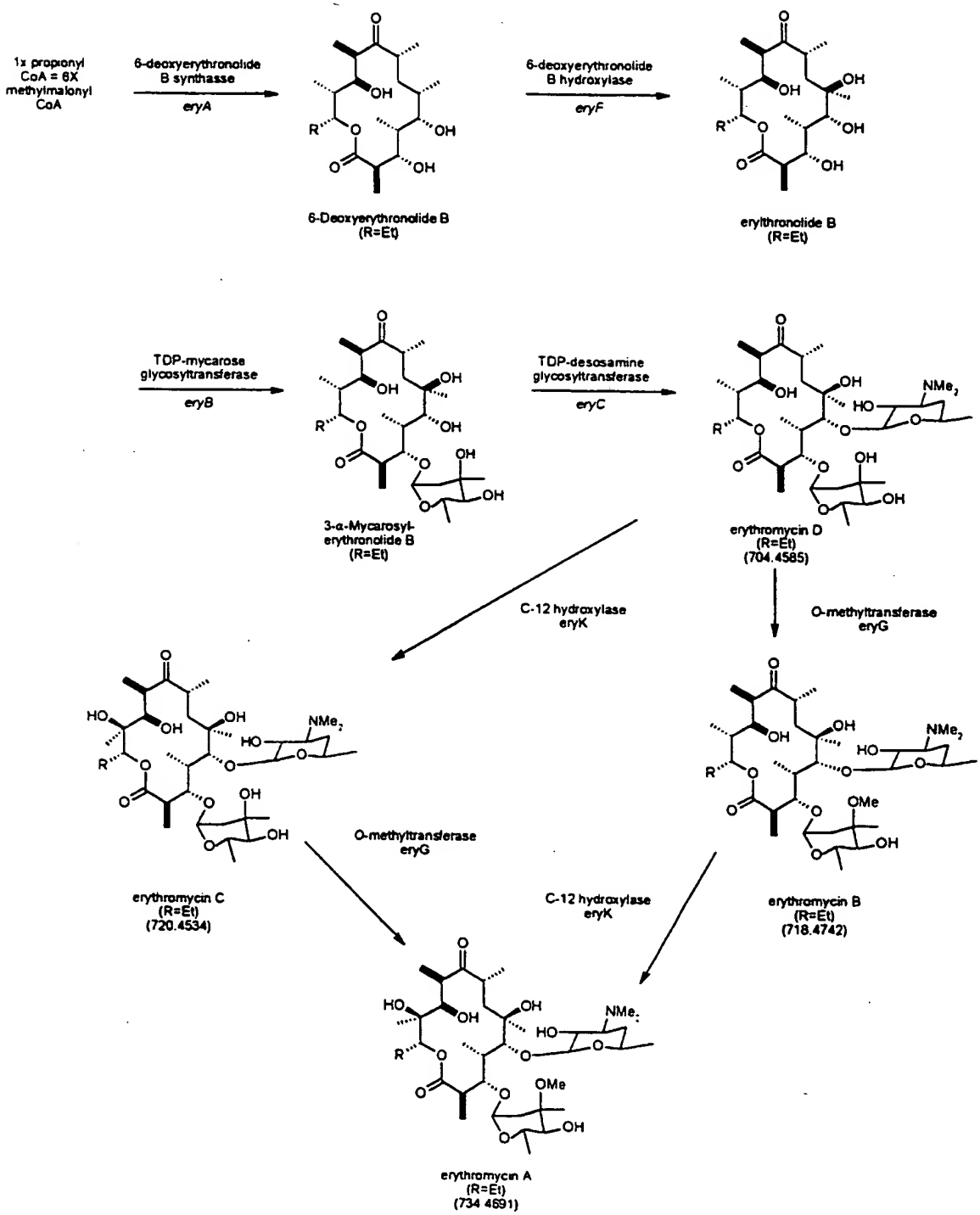

1x propionyl CoA + 6X methylmalonyl CoA
6-deoxyerythronolide B synthasse
eryA
6-Deoxyerythronolide B (R=Et)
6-deoxyerythronolide B hydroxylase
eryF
erylthronolide B (R=Et)
TDP-mycarose glycosyltransferase
eryB
3-α-Mycarosyl-erythronolide B (R=Et)
TDP-desosamine glycosyltransferase
eryC
erythromycin D (R=Et) (704.4585)
C-12 hydroxylase eryK
O-methyltransferase eryG
erythromycin C (R=Et) (720.4534)
O-methyltransferase eryG
C-12 hydroxylase eryK
erythromycin B (R=Et) (718.4742)
erythromycin A (R=Et) (734.4691)

FIG. 2b

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00117

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/76 C12N1/21 C07D493/08
//(C07D493/08,321:00,311:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 93 13663 A (ABBOTT LABORATORIES) 22 July 1993 cited in the application | 7 |
| A | see page 2, line 17 - page 3, line 2 see page 5, line 35 - page 7, line 18; examples 28-44 | 1-6,8,9 |
| | --- | |
| X | ANDREW F.A. MARSDEN ET AL.: "Engineering broader specificity into an antibiotic-producing polyketide synthase" SCIENCE, vol. 279, no. 5348, 9 January 1998, pages 199-201, XP002103747 LANCASTER, PA US cited in the application | 7 |
| A | see the whole document | 1-6,8,9 |
| | --- | |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 May 1999 | 25/06/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Montero Lopez, B |

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Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Inter >nal Application No
PCT/GB 99/00117

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 0 560 147 A (MEIJI SEIKA KAISHA LTD.)<br>15 September 1993<br>see page 3, line 34 - page 4, line 28 | 7,8 |
| A | LEONARD KATZ: "Manipulation of modular polyketide synthases"<br>CHEMICAL REVIEWS,<br>vol. 97, no. 7, 1997, pages 2557-2575,<br>XP002103748<br>cited in the application<br>see page 2565, right-hand column, paragraph 4<br>see page 2573, left-hand column, paragraph 2 - right-hand column, paragraph 4<br>see page 2574, left-hand column, last paragraph | |
| A | AKIO KINIMAKI ET AL.: "Mass spectrometry of platenolides and their derivatives in connection with structure elucidation"<br>JOURNAL OF ANTIBIOTICS,<br>vol. XXIX, no. 11, 1976, pages 1209-1217,<br>XP002103749<br>TOKYO JP<br>see figure 1A | 9 |
| P,X | WO 98 01546 A (BIOTICA TECHNOLOGY LIMITED)<br>15 January 1998<br>see page 6, paragraph 2 - page 7, paragraph 1<br>see page 10, paragraph 2 - paragraph 4<br>see page 14, paragraph 2 - page 16, paragraph 2 | 1,3-7 |

1

# INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter ...nal Application No
PCT/GB 99/00117

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9313663 A | 22-07-1993 | CA 2100791 A | 18-07-1993 |
| | | AU 665526 B | 11-01-1996 |
| | | AU 1245092 A | 03-08-1993 |
| | | EP 0626806 A | 07-12-1994 |
| EP 560147 A | 15-09-1993 | JP 6009679 A | 18-01-1994 |
| | | DE 69321632 D | 26-11-1998 |
| | | EP 0852231 A | 08-07-1998 |
| | | ES 2124267 T | 01-02-1999 |
| | | US 5444174 A | 22-08-1994 |
| WO 9801546 A | 15-01-1998 | AU 3450997 A | 02-02-1998 |
| | | AU 3451497 A | 02-02-1998 |
| | | EP 0909327 A | 21-04-1999 |
| | | EP 0910633 A | 28-04-1999 |
| | | WO 9801571 A | 15-01-1998 |
| | | GB 2331518 A | 26-05-1999 |
| | | AU 7666198 A | 30-12-1998 |
| | | WO 9854308 A | 03-12-1998 |